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Sulphide production and corrosion in seawaters during exposure to FAME diesel

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Experiments were designed to evaluate the corrosion-related consequences of storing/transporting fatty acid methyl ester (FAME) alternative diesel fuel in contact with natural seawater. Coastal Key West, FL (KW), and Persian Gulf (PG) seawaters, representing an oligotrophic and a more organic- and inorganic mineral-rich environment, respectively, were used in 60 day incubations with unprotected carbon steel. The original microflora of the two seawaters were similar with respect to major taxonomic groups but with markedly different species. After exposure to FAME diesel, the microflora of the waters changed substantially, with Clostridiales (Firmicutes) becoming dominant in both. Despite low numbers of sulphate-reducing bacteria in the original waters and after FAME diesel exposure, sulphide levels and corrosion increased markedly due to microbial sulphide production. Corrosion morphology was in the form of isolated pits surrounded by an intact, passive surface with the deepest pits associated with the fuel/seawater interface in the KW exposure. In the presence of FAME diesel, the highest corrosion rates measured by linear polarization occurred in the KW exposure correlating with significantly higher concentrations of sulphur and chlorine (presumed sulphide and chloride, respectively) in the corrosion products.

Keywords: seawater; carbon steel; FAME diesel; sulphide; microbiologically influenced corrosion

Introduction

Petroleum-derived diesel (petrodiesel) is often exposed to sulphate-containing water during transportation and storage, as for example, when seawater is used in ballast tanks (Little and Lee 2007). A vast literature has accumulated on the subject of microbiologically influenced corrosion (MIC) resulting from petrodiesel/seawater exposures (May and Neihof 1981; Neihof and May 1983; Chesneau and Dorris 1988; Shennan 1988; Gaylarde et al. 1999). Attention has focused on sulphate-reducing bacteria (SRB) as the major causative organisms in MIC due to production of sulphide. The introduction of plant-derived alternative diesel raises new questions about the microorganisms and their activities that foster MIC when such fuels are used. Do plant-derived diesels accelerate microbial sulphide production in a manner similar to petro diesel? Do characteristic corrosion-producing microbial communities develop in seawater exposed to alternative diesel fuels, regardless of the origin of the seawater? Does the brine/seawater chemistry, specifically the initial concentration of chloride, sulphate, and organic carbon, determine the impact of MIC? Experiments described in this paper were designed to provide quantitative information related to the factors affecting MIC of carbon steel in fatty acid methyl ester (FAME) diesel/seawaters exposures, by contrasting two seawaters with known differences in corrosion-related parameters after exposure to FAME diesel (Lee et al. 2007, 2010a).

FAME diesel is a renewable fuel produced from vegetable oils made by converting triglyceride oils to methyl (or ethyl) esters by transesterification (von Wedel 1999). FAME diesel does not contain sulphur and is characterized by the number of carbon atoms in the molecule, eg FAME C17. FAME diesel is a far less complex chemical mixture than petrodiesel, a factor that would be expected to influence both the selection of the microbial species involved in FAME diesel metabolism and the rate of biodegradation. However, it is unlikely that neat FAME diesel will be used routinely as an alternate fuel because of storage instability and gel formation at low temperatures (Kegl 2008). Specific properties vary depending on the type of oil from which the biodiesel was made (Knothe 2004; Barabas and Todorut 2011). FAME diesel mixes easily with petroleum diesel (Chotwichien et al. 2009) and the resulting blends offer environmental and operational benefits,

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including increased cetane (Rahimi et al. 2009) and lubricity (Anastopoulos et al. 2002; Knothe and Steidley 2005), as well as lower emissions (Lapuerta et al. 2008; Zheng et al. 2008).

Microbial growth in fuels is often limited by water availability and concentrated at fuel/water interfaces. FAME diesel is relatively hygroscopic and can absorb moisture from the atmosphere. Diesel methyl esters are sparingly soluble in seawater, with a saturation concentration of seven parts per million (ppm) at 17°C (von Wedel 1999). These fuel components are readily biodegraded under a variety of aerobic or anaerobic conditions (Aktas et al. 2010). The half-life for the biodegradation of vegetable methyl esters in agitated San Francisco Bay seawater was less than 4 days at 17°C (von Wedel 1999, 2000).

Two coastal seawaters representing two potentially different microbial and chemical environments were selected for the sulphide production and corrosion studies. Key West, FL, (KW) seawater is typical of open-ocean oligotrophic water. In the Persian Gulf (PG), total evaporation exceeds fresh water input, resulting in the concentration of dissolved species relative to those deemed characteristic of typical seawater (Bashitialshaaer et al. 2009). Lee et al. (2007) compared the chemistry and microflora of the two natural coastal seawaters when held under identical conditions with final SRB populations of 10^1–10^2 cells ml^{-1} (stirred and stagnant maintained in an anaerobic hood). The authors demonstrated that, despite higher level of initial sulphate and total organic carbon in the PG seawater, dissolved sulphide concentrations orders of magnitude higher were measured in KW seawater (eg 100 ppm *vs* 0.01 ppm). The authors concluded that it was not possible to predict the potential for microbiologically influenced sulphide induced corrosion based on dissolved sulphate and organic carbon concentrations in seawater. Lee et al. (2007) did not measure any parameter directly related to corrosion and the authors acknowledged the limitations of the microbial cultivation techniques.

In the current experiments, the microbial community in the seawaters at the start and conclusion of a 60 day exposure to FAME diesel was assessed using pyrosequencing of 16S rRNA gene sequences and the abundance of SRB and methanogens was determined by quantitative PCR (q-PCR) of functional genes essential for sulphate-reduction and methanogenesis. The corrosion behaviour of unprotected carbon steel exposed to FAME diesel/seawater (KW and PG) incubations held under anaerobic conditions was monitored using linear polarization techniques. Unprotected carbon steel, a common material used for fuel storage and seawater compensated ballast tanks, was used in the study.

Materials and methods

FAME diesel

A previously characterized soy-based diesel was obtained from US Navy Fuel and Lubes, Patuxent River, MD (Lee et al. 2009). The major components were FAME C17 and C19.

Seawater

The PG seawater was collected from the US Navy pier at Mina Sulman, Bahrain, while the KW seawater was collected at the Naval Research Laboratory (NRL) Marine Corrosion Facility, Key West, FL. PG seawater was collected within the first meter of surface seawater by hand immersing each collection container underwater. KW seawater was collected at a depth of 1.2–1.5 m by intake pipes powered by suction pumps. Seawaters were shipped with no headspace to the NRL, Stennis Space Center (NRLSSC), MS, in three 19 l opaque plastic containers. The pH and salinity (parts per thousand [ppt]) were recorded immediately. The two seawaters have been described in more detail elsewhere (Lee et al. 2004, 2005, 2006, 2007; Ray et al. 2005).

Samples of both seawaters were evaluated for the rates of *in situ* sulphate reduction using a radiotracer technique (Ulrich et al. 1997). Samples (10 ml) were dispensed into sterile anoxic serum bottles flushed with nitrogen and supplemented with 2 μCi $Na_2{}^{35}SO_4$ (698 mCi ml^{-1}, MP Biomedicals, Inc. Irvine, CA) per bottle. Inorganic sulphur was extracted by a chromium reduction technique and quantified (Ulrich et al. 1997). Filter-sterilized water samples (0.22 μm) were used as negative controls. Incubations with 10 mM sodium lactate, a common substrate for cultivation of SRB or 1 ml of sterile crude oil (Bebee oil field, OK) were used as positive controls. An oil-degrading SRB, *Desulfoglaeba alkanexedens* strain Lake (Davidova et al. 2006), was used as an inoculum with the crude oil amendment. Positive controls were used to assess the presence of substances (eg inhibitors, toxicants) in the seawaters that might predispose the sulphate reduction assay to failure. Sulphate was analyzed by ion chromatography (Gieg et al. 2008).

Metal coupons

Coupons of UNS C10200 carbon steel (CS) (0.20% C, 0.47% Mn, 0.012% P, 0.013% Si, bal. Fe) were fabricated (Metal Samples, Munford, AL) to dimensions of 1.5875 cm diameter and 0.3175 cm thick. A wire was attached to the backside of the coupons with carbon tape to provide an electrical connection. Coupons were individually mounted in EpoThin™

epoxy (Buehler Ltd, Lake Bluff, IL) to isolate the wire connection and to establish an exposed area of 2 cm^2. Wired/mounted coupons (electrodes) with as-mill finish were sonicated in liquid detergent, rinsed successively with acetone, ethanol and distilled water and dried with nitrogen (N_2) gas.

Exposure conditions

Two incubation chambers were constructed to expose CS electrodes to seawater and FAME biodiesel. The chambers were cylindrical (35.5 cm diameter and 27.9 cm height), constructed from heavy gauge, chemical resistant, opaque black plastic and sealed with a lid compressed onto a rubber gasket (Lee et al. 2004, 2005, 2006; Ray et al. 2005). Ag/AgCl electrodes (Electrochemical Devices, Inc., Albion, RI, model IP) and Pt/Nb mesh (Intrepid Industries, Inc., Lebanon, NJ) were used as reference and counter electrodes, respectively. For each chamber, nine CS electrodes were arranged vertically so that triplicate coupons were exposed to three conditions (from top to bottom): (1) FAME diesel, (2) FAME diesel/seawater interface, and (3) seawater. An Oxyguard® stationary dissolved oxygen (DO) probe was placed at the bottom of the chamber. Chambers were filled with 4 l of seawater (one with KW and one with PG seawater), amended with 6 l of FAME diesel, and placed in an anaerobic hood with an atmosphere of 0.01% CO_2, 10% H_2 and the balance N_2. The CO_2 concentration was chosen to maintain a seawater pH of between 7.8 and 8.2 (Lee et al. 2010b). Anaerobic hood temperature was maintained at 23°C. Chambers and electrodes were cleaned with soapy water and ethanol prior to introduction of fuel and water.

The pH and salinity were recorded after 60 days. The DO concentration was monitored continuously at 2-h intervals by a Madgetech, Inc. (Warner, NH) mini-data logger (Model Volt101) attached to the probe. The DO probe resolution was 0.1 ppm. The sulphide concentration was measured after the 60 days exposure using the methylene blue method (Clesceri et al. 1998) with a commercial CHEMets® analysis kit (model K-9510).

DNA extraction and quantitative polymerase chain reaction (qPCR) amplification

DNA was extracted from 1 l of freshly collected filtered seawater (KW, PG) using a bead-beating protocol (MegaPrep Ultra Clean DNA Extraction Kit, MO BIO Laboratories, Inc., Carlsbad, CA), concentrated by ethanol precipitation, following the manufacturer's directions, and resuspended in PCR grade water. The DNA was extracted from 600 ml of filtered FAME diesel/seawater incubations (KWBD, PGBD) after 60 days using a bead-beating protocol (PowerSoil DNA Extraction Kit, MO BIO Laboratories, Inc., Carlsbad, CA) and resuspended in PCR grade water.

Estimates of the number of bacterial and archaeal 16S rRNA genes were made using qPCR. The numbers of eubacteria and archaeal were assayed using the primers 27F and 338R, or A8F and 344R, respectively, as previously described (Stevenson et al. 2011). The number of copies of *mcrA* (coding for subunit *a* of methyl-S-CoM methylreductase, an essential enzyme in methanogenesis) was assayed in order to estimate the numbers of methanogens, using primers mlas and mcrA-rev (Steinberg and Regan 2008). Estimates of the number of copies of a gene coding for an enzyme essential for sulphate reduction, dissimulatory (bi)sulphite reductase (*dsrA*), were assayed with primers dsr1F and dsr5R (Wagner et al. 1998; Karr et al. 2005). The number of copies of a second gene coding for an enzyme essential for sulphate reduction, adenosine-5′-phosphosulphate reductase (*apsA*), were assayed with the primers RH1apsF and RH2apsR (Ben-Dov et al. 2007).

Thermal cycling, data acquisition and analyses were carried out with the StepOnePlus™ Real-Time PCR System and StepOne Software v2.1 (Life Technologies, Carlsbad, CA). Cycling conditions for bacterial and archaeal 16S rRNA were described in the references in the previous paragraph. For each qPCR run, a 1:10 dilution series of a control DNA plasmid containing a bacterial, archaeal 16S rRNA gene sequence, *dsrA*, *apsA*, or *mcrA* gene sequence was used to generate a 5–7 point standard curve. Standards and samples were assayed in triplicate.

Bacterial 16S rRNA gene amplification and pyrosequencing analysis

16S rRNA gene libraries were generated using end-point PCR with the eubacterial 16S rRNA primers 27F and 338R, as per Stevenson et al. (2011). Duplicate reactions were pooled, purified and concentrated using Amicon Ultra 30K filtration devices (Millipore). Titanium Fusion A and B primers, TiA-8nt-27F and TiB-338R from Stevenson et al. (2011), with unique 8nt tags for each library were added by amplification for six cycles. Tagged libraries were purified, quantified by fluorometry (Invitrogen), and equimolar amounts of each were combined and pyrosequenced using the GS-FLX instrument (454 Life Sciences/Roche) at the Advanced Center for Genome Technology (OU, Norman, OK).

Sequences were analyzed using the bioinformatics software package, mothur (Schloss et al. 2009). An

implementation of the Amplicon Noise algorithm (Quince et al. 2011) was used to reduce sequencing error. Sequences containing ambiguities, homopolymers greater than eight, or errors in the forward primer or barcode were removed. Unique sequences were trimmed to overlap a minimum of 200 bp and aligned against the SILVA reference alignment database (Prucsse et al. 2007) using the NAST-aligner (DeSantis et al. 2006). Sequences were pre-clustered (Huse et al. 2010) and screened for chimeras using UChime (Edgar et al. 2011). A distance matrix was generated and used to cluster sequences into operational taxonomic units (OTUs) at the 97% similarity level using the average neighbor algorithm (Huse et al. 2010). A representative sequence from each OTU was assigned a taxonomic classification based on the naïve Bayesian classifier (Wang et al. 2007) and all richness and diversity measurements were calculated using the mothur software package (Schloss et al. 2009). Sequences were deposited in the short read archive (SRA) of GenBank under accession numbers SRX115023-SRX-115026.

*Clone library creation and sequencing of alkane 1-monooxygenase (*alkB*)*

Degenerate primers targeting *alkB* (a catalytic subunit of alkane 1-monooxygenase) (Kloos et al. 2006), were used to create libraries of a common aerobic degradative pathway gene. Clone libraries for the FAME diesel/seawater incubations were created using the TOPO TA Cloning Kit (Invitrogen Corp., Carlsbad, CA). Sequencing was performed on an ABI model 3730 capillary sequencer (Microgen: The Laboratory for Genomics and Bioinformatics, Oklahoma City, OK). Sequencher (Gene Codes Corp. Ann Arbor, MI) was used to cluster the sequences into 95% similarity level. BLASTN searches (Altschul et al. 1990) and the representative sequences were compared to the GenBank database. Representative sequences were translated (EMBOSS Transeq) (Rice et al. 2000), compared to the protein database using BLASTP 2.2.26 (Altschul et al. 1997) and conserved protein domain homologies assessed with the Batch Web CD-Search Tool (Marchler-Bauer et al. 2009). Representative sequences were submitted to GenBank and received accession numbers JQ279685-JQ279689.

Electrochemical methods

Computer-controlled Gamry Instruments (Warminster, PA) potentiostat (model PCI4^{TM}) and multiplexer (model ECM8^{TM}) were used for all electrochemical measurements. The corrosion potential (E_{corr}) [$V_{Ag/AgCl}$] and polarization resistance (R_p) [ohms cm^2] were determined by the linear polarization resistance technique (LPR) (Scully 2000) on each electrode once a day. Current density (i) [A cm^{-2}] was recorded as the potential of each electrode was scanned from -10 mV to $+10$ mV *vs* E_{corr} at the ASTM Standard G5-94 (2004) scan rate of 0.1667 mV s^{-1} (0.6 V h^{-1}). Compensation for solution resistance (R_s) was not necessary because of the relatively high conductivity of seawater (Wiesenburg and Little 1988) and low current densities (μA cm^{-2}). The reciprocal of R_p (R_p^{-1}) [ohms^{-1} cm^{-2}] is proportional to the instantaneous corrosion rate.

Post-exposure metal surface examination

After the 60 days exposure, electrodes were removed and imaged using a Nikon macro digital camera (model S-700). Each electrode was fixed in cacodylate buffered 4% glutaraldehyde in artificial seawater and rinsed in distilled water. Corrosion morphology and corrosion product elemental composition were characterized with environmental scanning electron microscopy (ESEM) and energy dispersive spectroscopy (EDS), respectively (Ray and Little 2003). Coupons were removed from epoxy mounts, the wires disconnected, acid cleaned to remove corrosion products according to ASTM Standard G1-03 (2003), and re-examined with ESEM. Coupons were scanned using a Nanovea (Irvine, CA) non-contact optical profiler (model PS50) with a 400 μm optical laser pen to reconstruct high contrast 3-D digital images. Pit depths were measured from these reconstructed images.

Results

Seawater

In both KW and PG seawaters, DO decreased from 4.8 ppm to <0.1 ppm (the detection limit) after 1 day and remained at that level over the exposure period. At the onset of the experiments, the pH values of KW and PG seawaters were 8.41 and 8.37, respectively. PG seawater had a higher salinity (44 ppt) than KW seawater (39 ppt). After 60 days exposure to FAME diesel, the salinities remained unchanged for all conditions and the pH values of KW and PG seawaters were 7.58 and 7.10, respectively. At the end of the incubation, the dissolved sulphide for anaerobic KW and PG seawaters was 6 and 2 ppm, respectively. Seawater *in situ* rates of sulphate reduction were low, barely above background detection levels (Table 1) and they did not change significantly with addition of crude oil. However, amendment of the incubations with an easily degradable carbon source, lactate (10 mM), resulted in a 10-fold increase in sulphate reduction in KW seawater and about a 2-fold increase in PG seawater. Incubations amended with crude oil

and inoculated with *D. alkanexedens* showed a 10–20 fold increase in sulphate reduction compared to that of the unamended seawater.

DNA extraction and quantitative polymerase chain reaction (qPCR) amplification

Assuming equal DNA extraction and PCR amplification efficiency for all four samples, the KW and PG seawater samples contained approximately equal numbers of the bacterial 16S rRNA gene sequence, while the FAME diesel/seawater incubations (KWBD, PGBD) contained roughly one hundred-fold fewer copies (Table 2). The numbers of two essential genes for sulphate-reduction, *dsrA* (codes for dissimulatory (bi)sulphite reductase) and *apsA* (codes for adenosine-5′-phosphosulphate reductase) were below the level of detection (<10 copies ml^{-1} sample) in most samples, suggesting that very few SRB were present either in the original seawater or after incubation with FAME diesel. Archaeal 16S rRNA gene sequences were detected in both seawater samples but at much lower numbers than bacterial 16S rRNA sequences. Methanogenic archaea were detected in all samples with the highest abundance in KW. This determination was based on quantification of a gene coding for an enzyme essential for methanogenesis, *mcrA* (subunit *a* of methyl-S-CoM methylreductase). Methanogens increased in relative abundance, though not in absolute numbers, when seawater was incubated with FAME diesel.

Table 1. Rates of sulphate reduction activity (SRA) in the seawater samples.

Sample	Persian Gulf seawater (SRA μmol $S\ l^{-1}\ day^{-1}$)	Key West seawater (SRA μmol $S\ l^{-1}\ day^{-1}$)
In situ (no additions)	11.96 ± 1.3	17.7 ± 3.3
Amended with lactate	23.5 ± 1.7	115
Amended with crude oil*	10.3 ± 2.3	13.95 ± 0.8
Amended with crude oil and inoculated**	155 ± 6.7	264 ± 40
Sterile control	7.95 ± 1.7	7.5 ± 3.5

Note: *Sterile crude oil; **_Desulfoglaeba_ strain Lake, an alkane-degrading SRB (Davidova et al. 2006).

Bacterial 16S rRNA gene amplification and pyrosequencing analysis

Pyrosequencing libraries of 16S rRNA gene sequences were constructed for the original seawater samples (KW and PG) and those seawaters incubated with FAME diesel/seawater (KWBD, PGBD). The number of sequences obtained ranged between 8726 and 13308, while the number of OTUs, formed by clustering the sequences at a sequence similarity of 97%, ranged from 945 (KW) to 235 (PGBD). KW seawater had the highest bacterial community diversity, followed by PG seawater, then KWBD, with PGBD the least diverse, based on calculation of three different diversity/richness indices (Shannon, ACE, Chao1). The 95% confidence intervals for the ACE and Chao1 diversity indices values did not overlap. Taxonomic classification of the sequences demonstrated striking differences in the relative proportions of different bacterial groups between the original seawaters and those seawaters after incubation with FAME diesel (Figure 1). The three major groups in the natural seawaters were Bacteroidetes, Alphaproteobacteria, and Gammaproteobacteria, as is typical for marine samples (Kirchman 2002). The relative proportion of Bacteroidetes and Alphaproteobacteria decreased in the FAME diesel incubations whereas Firmicutes (primarily Clostridiales) and Gammaproteobacteria increased. The FAME diesel incubations diverged, with KWBD containing ~18% sequences affiliated with candidate division OP1, 6.7% Tenericutes (formerly Mollicutes), and approximately half the proportion of Firmicutes found in PGBD. Very few sequences were affiliated with SRB. Deltaproteobacteria comprised <1% of any of the four samples, three sequences were affiliated with Thermodesulfobacteria, and none with the Gram-

Table 2. Estimates of the number of different cell types based on q-PCR analyses.

Estimates from qPCR	KW*	PG	KWBD	PGBD
Bacterial cells ml^{-1}	2.75×10^7	2.66×10^7	4.97×10^5	1.72×10^5
Dsr-bearing cells ml^{-1}**	3.17	BDL	BDL	BDL
Aps-bearing cells ml^{-1}***	BDL	BDL	BDL	BDL
Archaeal cells ml^{-1}	3.05×10^3	2.19×10^3	BDL	BDL
Mcr-bearing cells ml^{-1}****	2.48×10^3	25	121	47.4

Note: *KW: Key West seawater; PG: Persian Gulf seawater; KWBD: FAME diesel incubated with KW seawater; PGBD: FAME diesel incubated with PG seawater. **Dsr-bearing cells: cells that contain a copy of the gene coding for dissimulatory (bi)sulphite reductase, eg SRB. ***Aps-bearing cells: cells that contain a copy of the gene coding for adenosine-5′-phosphosulphate reductase, eg SRB. ****Mcr-bearing cells: cells that contain a copy of the gene coding for subunit *a* of methyl-S-CoM methylreductase, eg methanogens. BDL: below detection limits.

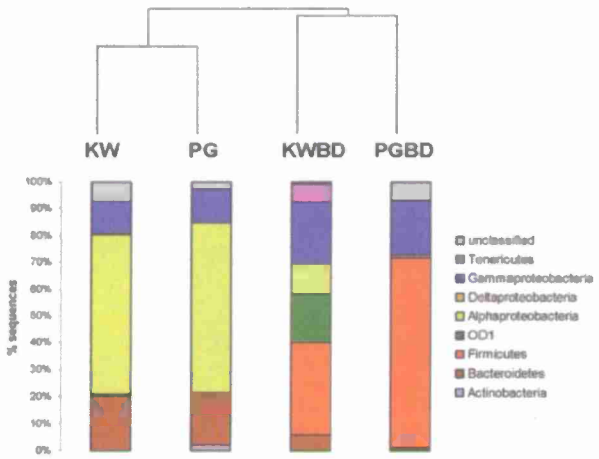

Figure 1. Analysis of bacterial 16S rRNA gene libraries created by pyrosequencing. Top: dendrogram showing similarity among natural seawater samples (KW, PG) and FAME diesel/seawater incubations (KWBD, PGBD) based on a measurement of community structure (θ_{YC}) (Yue and Clayton 2005). Bottom: relative abundances of sequences at the Phylum level (Proteobacteria represented as Classes). Analyses were performed using the mothur software package (Schloss et al. 2009).

positive SRB genera *Desulfotomaculum*, *Desulfosporomusa*, or *Desulfosporosinus*.

Although the relative proportion of major taxonomic groups of bacteria was similar in the seawaters, the samples from two of these groups differed when examined at a finer scale. Two Orders, Sphingobacteriales and Flavobacteriales, together with unclassified Bacteroidetes, made up the majority of sequences affiliated with Bacteroidetes. Approximately 75% of the KW Bacteroidetes sequences were classified as Flavobacteriales and 3% as Sphingobacteriales, while in the PG sample Flavobacteriales made up 30% and Sphingobacteriales 26% of the Bacteroidetes. Proportionately more members of the Sphingobacteriales in more nutrient-rich marine waters has been observed previously in comparing samples from the North Atlantic (Gomez-Pereira et al. 2010). The Alphaproteobacteria of KW were more evenly divided between Rhodobacteriales and Rickettsiales than the PG sample, which was dominated by SAR11 clade sequences. The SAR11 clade sequences (cultivated isolate: *Pelagibacter ubique*) are typically abundant in seawater (Giovannoni et al. 1990; Schattenhofer et al. 2009). However, the dominant SAR11 OTU found in KW was not found in PG, which contained its own abundant SAR11 OTU. Sequences were affiliated with five different orders of Gammaproteobacteria but the majority of sequences could not be classified within a particular Order of Gammaproteobacteria. Comparison at an even finer scale revealed that the number of OTUs (97% level of similarity) common to both KW and PG was only 73 (KW: 945 OTUs in total, PG: 431 OTUs in total). A difference at a fine scale was also true for the FAME diesel incubations; the number of OTUs (97% level of similarity) common to both KWBD and PGBD was 29 (KWBD: 287 OTUs in total, PGBD: 235 OTUs in total).

Sequences affiliated with Clostridiales were in low abundance in seawater (KW: 5 sequences, PG: 1 sequence) but increased in relative abundance in the FAME diesel/seawater incubations (KWBD: 4520 sequences, PGBD: 6646 sequences). Although 300 OTUs were classified as Clostridiales, 253 of these contained 1–10 sequences, and 43 contained 12–284 sequences, which collectively comprised 20% of the Clostridiales sequences in KWBD and 35% in PGBD. The four dominant OTUs tended to occur in either KWBD or in PGBD exclusively (GJKYC, GJ15XN) or were more abundant in one sample than the other (G46TI, I8UGB). GJKYC had 97–98% similarity to 16S rRNA gene sequences of five cloned sequences from ocean sediments (GQ267079, GQ267083, GQ267129, GQ267134) or coral (AF365717) and from *Clostridum* sp. DY192 (HQ696463), which was isolated from Indian Ocean sediments (unpublished). The closest match to a named species was to *Clostridium halophilum* strain DSM 5387 (accession # X77837, 90%). GJ15XN, the representative sequence of the most abundant OTU in PGBD, was not detected in the other three samples. GJ15XN had low similarity (90–94%) to various sequences cloned from other environments such as corals, lakes, and an anoxic sea basin. The G46TI represented 675 sequences found in KWBD and 137 sequences from PGBD. It was most similar (96%) to a sequence cloned from a sample obtained from a borehole deep within a gold mine (accession # AF459050) (Baker et al. 2003). The similarity of G46TI to the16S rRNA gene sequence of any isolate was <90%. The I8UGB represented the second most abundant OTU in KWBD (most abundant was similar to OD1) and also was represented in PGBD. The 8UGB was 96% similar to the 16S sequence of *Sporosalibacterium faouarense* SOL3f37 (accession # EU567322), which had been isolated from oil-contaminated soil (Rezgui et al. 2011). *S. faouarense* SOL3f37 does not use sulphate, thiosulphate, or elemental sulphur as electron acceptors and was not tested for growth on hydrocarbons.

Sequences similar to those of eight genera containing hydrocarbonoclastic strains were in low abundance in the original seawater samples (Table 3). However, sequences highly similar to those of *Marinobacter* (Gammaproteobacteria) increased in relative abundance to become 10–20% of the total number of sequences in FAME diesel/seawater incubations. A

total of 56 OTUs (97% level of similarity) were classified as *Marinobacter*, but 48 contained 1–7 sequences, and 6 others 11–171 sequenees each, 15% or less of the total *Marinobacter* sequenees. The two dominant *Marinobacter* OTUs occurred differentially in the FAME diesel/seawater incubations. JPO7T represented 998 sequences in KWBD and none in PGBD, while JAFLS represented 241 sequences in KWBD and 1632 in PGBD. The sequence of JPO7T matched (99%) to that of *Marinobacter hydrocarbonoclasticus* isolate MARC4F (accession # DQ768638) (Cui et al. 2008) and >30 other sequences considered similar to those of *M. hydrocarbonoclasticus* strains. Thc sequencc of JAFLS matched 100% to that of *Marinobacter aquaeolei* VT8, isolated from an oil-producing well (accession # NR027551) (Huu et al. 1999) and that of more than two dozen *M. hydrocarbonoclasticus* strains.

*Clone library creation and sequencing of alkane 1-monooxygenase (*alkB*)*

Spccific primers were used to amplify sequences eoding for a portion of an enzyme that initiates aerobic hydroearbon degradation (*alkB*, alkane 1-monooxygenase) from the FAME diesel/seawater ineubations. The eloned sequenees (15 from KWBD, 14 from PGBD) were grouped into five OTUs with 95% nucleotide sequence similarity. The representative sequences were all affiliated with *alkB* gene sequences derived from well-known alkane-degrading marine *Marinobacter* (Gammaproteobacteria). The OTU representing the largest number of sequences (JQ279685, six sequenees from KWBD, 10 sequenees from PGBD), had 85% nucleotide sequenee similarity to *Marinobacter adhaerens* HP15 alkane 1-monooxygenase (CP001978) and 94% predicted amino acid similarity to *M. adhaerens* HP15 alkane 1-monooxygenase. Other high scquenee similarities for JQ279685 were to *M. hydrocarbonoclasticus* strain S17-4 (nucleotide, EU 853368), *M.* sp. P1-14D (amino aeid, ACS91349), *M. aquaeolei* VT8 (amino acid, YP960105), and *M.* sp. MnI7-9 (amino acid, ZP09160710). Representative sequences of the other four OTUs were most similar to the same group of *Marinobacter* strains, but two of the four OTUs had their highest nucleotide similarity (94%, 95%) to *M. hydrocarbonoclasticus* strain S17-4 or *M.* sp. P1-14D.

Table 3. Number of sequences classified as those of genera containing strains capable of degrading hydrocarbons.

Genus	KW	PG	KWBD	PGBD
Kordiimonas (α)*	14	0	9	0
Gaetbulibacter (Bact)**	4	1	0	0
Marinobacter (γ)***	0	1	1410	1918
Alcanivorax (γ)	4	9	0	0
Cycloclasticus (γ)	18	0	0	0
Alteromonas (γ)	4	10	2	5
Pseudomonas (γ)	1	1	0	0
Shewanella (γ)	0	2	0	0

*α: Alphaproteobacleria; **Bact: Bacteroidetes; ***γ: Gammaproteobacleria.

Macroscopic observations

After the 60 days exposure, the chambers were opened. The KW chamber had a very strong putrid, sulphide smell while the odor was less pronounced in the PG chamber. A viscous, oily, cloudy layer at the FAME diesel/seawater interface was observed in both ehambers, but was more pronounced in the KW seawater chamber. As shown in Figure 2, all electrodes

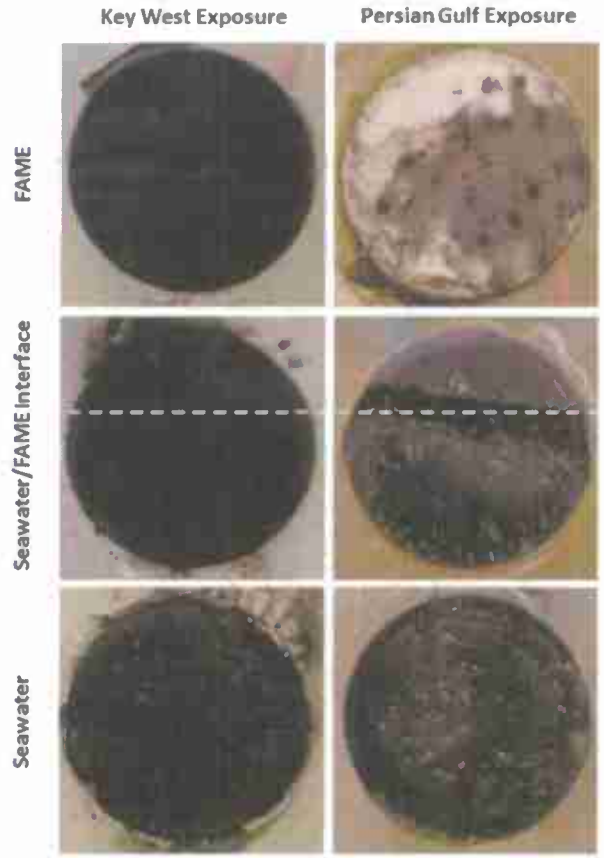

Figure 2. Carbon steel electrodes after 60 days exposure in KW (left column) and PG (right column) seawater with FAME diesel addition at different positions in the fuel/seawater mixture column. Top row: immersed in FAME diesel; middle row: FAME diesel/seawater interface; bottom row: immersed in seawater. Dashed line indicates the spatial relationship of the FAME diesel/seawater interface with the electrode face.

(independent of electrode position) exposed in the KW seawater with FAME diesel were completely black. For the PG exposure, electrodes immersed in FAME diesel retained the mill finish except for small areas (< 1 mm diameter) of black corrosion products. At the FAME diesel/seawater interface, differences between electrode regions exposed to diesel (top – dull gray) and seawater (bottom – black) were observed. Black corrosion products covered the entire surfaces of electrodes immersed in seawater.

Electrochemical behaviour

Figure 3 shows R_p^{-1} (ohms^{-1} cm^{-2}) as a function of the 60 day exposure period for CS electrodes exposed to FAME diesel and KW or PG seawater. Triplicate data were averaged for each electrode position, ie the FAME diesel/seawater interface and seawater. Collection of electrochemical data for coupons immersed in diesel was not possible due to the very high resistivity (megaohms) of the diesel layer. Electrode position did not affect corrosion rate above an order-of-magnitude for each exposure position. Initially, the corrosion rates for KW decreased from 10^{-4} to 10^{-6} ohms^{-1} cm^{-2} but at 12 days a sulphide odor was detected and the corrosion rate increased to 10^{-2} ohms^{-1} cm^{-2} by day 23. The PG incubation produced lower corrosion rates ($< 10^{-6}$ ohms^{-1} cm^{-2} average). The maximum standard deviations (SDs) of corrosion rates for KW and PG exposures were $\pm 10^{-5}$ and $\pm 10^{-7}$ ohms^{-1} cm^{-2}, respectively.

Figure 4 shows triplicate averaged E_{corr} ($V_{Ag/AgCl}$) as a function of time over the 60 day exposure period (same incubation conditions as in Figure 3). Throughout the exposure period, E_{corr} values in KW seawater were independent of electrode position and lower than E_{corr} values in PG seawater. Initially, the E_{corr} values in KW seawater for both positions was -0.750 $V_{Ag/AgCl}$, increased to -0.700 $V_{Ag/AgCl}$ after 12 days, and dropped to near -0.800 $V_{Ag/AgCl}$ after 23 days. In contrast to the KW exposure, the E_{corr} values in PG seawater were highly dependent on electrode position with the diesel/PG seawater interface position consistently being the higher of the two. After 1 day, the E_{corr} values for the PG seawater and FAME diesel/PG seawater interface positions were -0.700 $V_{Ag/AgCl}$ and -0.580 $V_{Ag/AgCl}$, respectively. At day 15, both positions dropped to -0.760 $V_{Ag/AgCl}$. The E_{corr} values for the PG seawater position slowly increased to -0.730 $V_{Ag/AgCl}$ over the remainder of the exposure period. The E_{corr} values for the diesel/PG seawater interface position increased steeply to -0.550 $V_{Ag/AgCl}$ by day 37, dropped to -0.675 $V_{Ag/AgCl}$ by day 41, and slowly increased to -0.620 $V_{Ag/AgCl}$ by the end of the exposure period. The maximum SDs of E_{corr} for KW and PG exposures were ± 0.010 and ± 0.025 V, respectively.

Corrosion product characterization

Accumulations of black precipitates were observed in both KW and PG waters supplemented with FAME diesel. The corrosion products in KW and PG seawaters consisted of small nodules and significant amounts of extracellular polymeric substances (EPS) (Figure 5). The presence of EPS was also apparent at the diesel/seawater interface. Sulphur (presumed

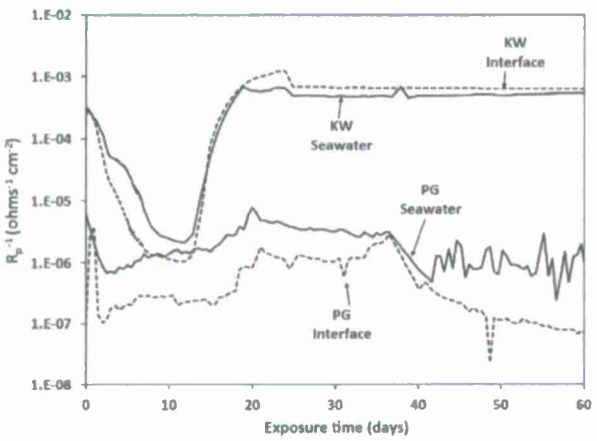

Figure 3. Average logarithmic inverse R_p instantaneous corrosion rates (ohms^{-1} cm^{-2}) of CS electrodes as a function of exposure time (days). Seawater = electrode immersed completely in seawater; interface = electrode at diesel/seawater interface.

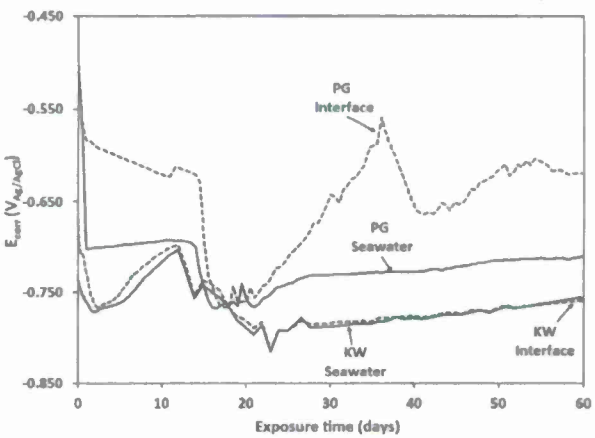

Figure 4. Average corrosion potential (E_{corr} *vs* $V_{Ag/AgCl}$) of CS electrodes as a function of exposure time (days). Seawater = electrode immersed completely in seawater; interface = electrode at diesel/seawater interface.

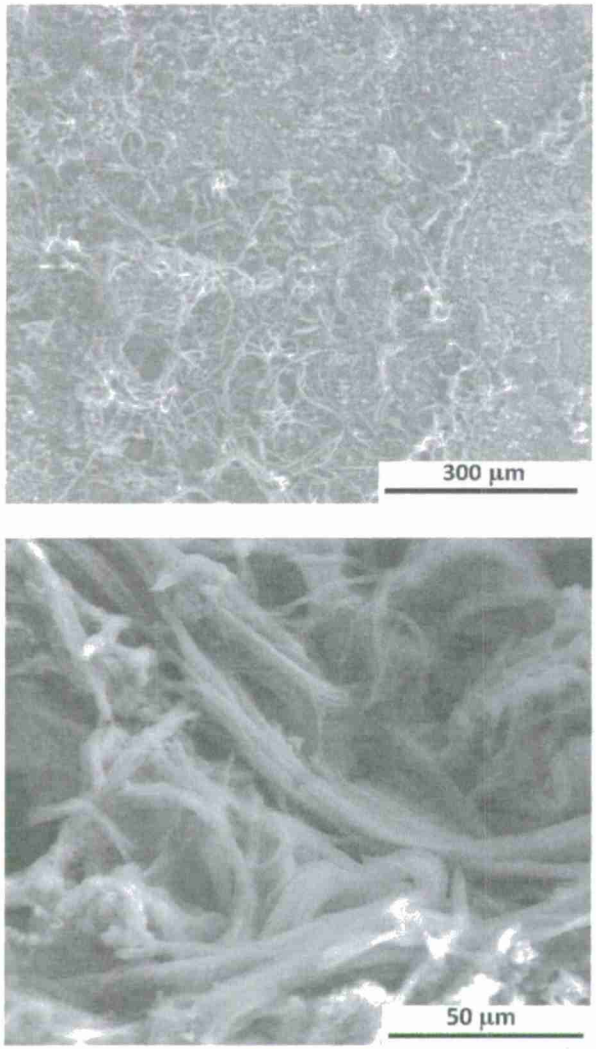

Figure 5. Corrosion products in PG (top) and KW (bottom) seawaters consisted of small nodules and significant amounts of extracellular polymeric substances.

sulphide) was detected on all electrodes irrespective of exposure position (Table 4). Significantly higher average sulphur concentrations were present in corrosion products from the KW seawater exposure (19–30 wt%) compared with the PG seawater (1–3 wt%). Chlorine (presumed chloride) was detected in corrosion products on all exposure conditions in KW seawater (4–7 wt%) while only the corrosion products in the FAME layer in the PG exposure had measureable chlorine concentration (< 1 wt%).

Corrosion morphology

Exposure to FAME diesel in the presence of KW seawater resulted in a mostly intact surface with pits ranging in diameters from 30 to 1000 μm (Figure 6). The deepest pits (155 μm) were detected at the FAME diesel/seawater interface. Fewer deep pits were observed above (75 μm) and below (35 μm) the interface. Exposure to KW seawater layer resulted in a larger number of pits than exposure to the FAME diesel layer.

Exposure to FAME diesel in the presence of PG seawater resulted in isolated pits (20 μm maximum depth) below the interface in the seawater layer. Small diameter (10 μm) hemispherical pits were not solely associated with the FAME diesel/PG seawater interface but were located over the entire electrode surface. Exposure in the FAME layer reproducibly resulted in an intact, passive surface with a few groupings of coalesced pits with maximum depths of 75 μm.

Table 4. Average sulphur and chlorine concentration (wt%) in corrosion products on carbon steel after 60 days exposure to seawater with FAME diesel addition.

Electrode position	Sulphur (wt%)		Chlorine (wt%)	
	KW	PG	KW	PG
FAME	19.7 ± 4.8	1.7 ± 0.02	4.5 ± 1.6	0.49
FAME/SW interface	23.6 ± 7.4	0.5 ± 0.5	4.7 ± 2.9	0
Seawater	30.2 ± 6.1	2.4 ± 0.5	6.6	0

Discussion

Differences in the chemistries and microflora of KW and PG seawaters have been examined in detail and reported elsewhere (Lee et al. 2007). Briefly, PG seawater has a higher salinity (44 ppt *vs* 39 ppt), more total organic carbon (1.94 ppm *vs* 1.79 ppm), more sulphate (4696 ppm *vs* 3864 ppm) and fewer microorganisms as measured in liquid culture than KW seawater. The higher concentrations of dissolved species have been attributed to high temperatures, evaporation and poor mixing in the area (Brewer and Dyrssen 1985). Despite the higher concentrations of chloride and sulphate in PG seawater, in the present study KW seawater with FAME diesel was more corrosive than PG seawater. The highest corrosion rate (R_p^{-1}) for KW with FAME diesel was 1×10^{-3} ohms^{-1} cm^{-2}, while R_p^{-1} for the PG seawater/FAME diesel exposure was $< 1 \times 10^{-6}$ ohms^{-1} cm^{-2}. The highest corrosion rate measured for anaerobic KW water without fuel (Lee et al. 2004) was more than an order of magnitude lower than the R_p^{-1} values measured in the present study.

After 60 days exposure to FAME diesel under anaerobic conditions, higher dissolved sulphides were

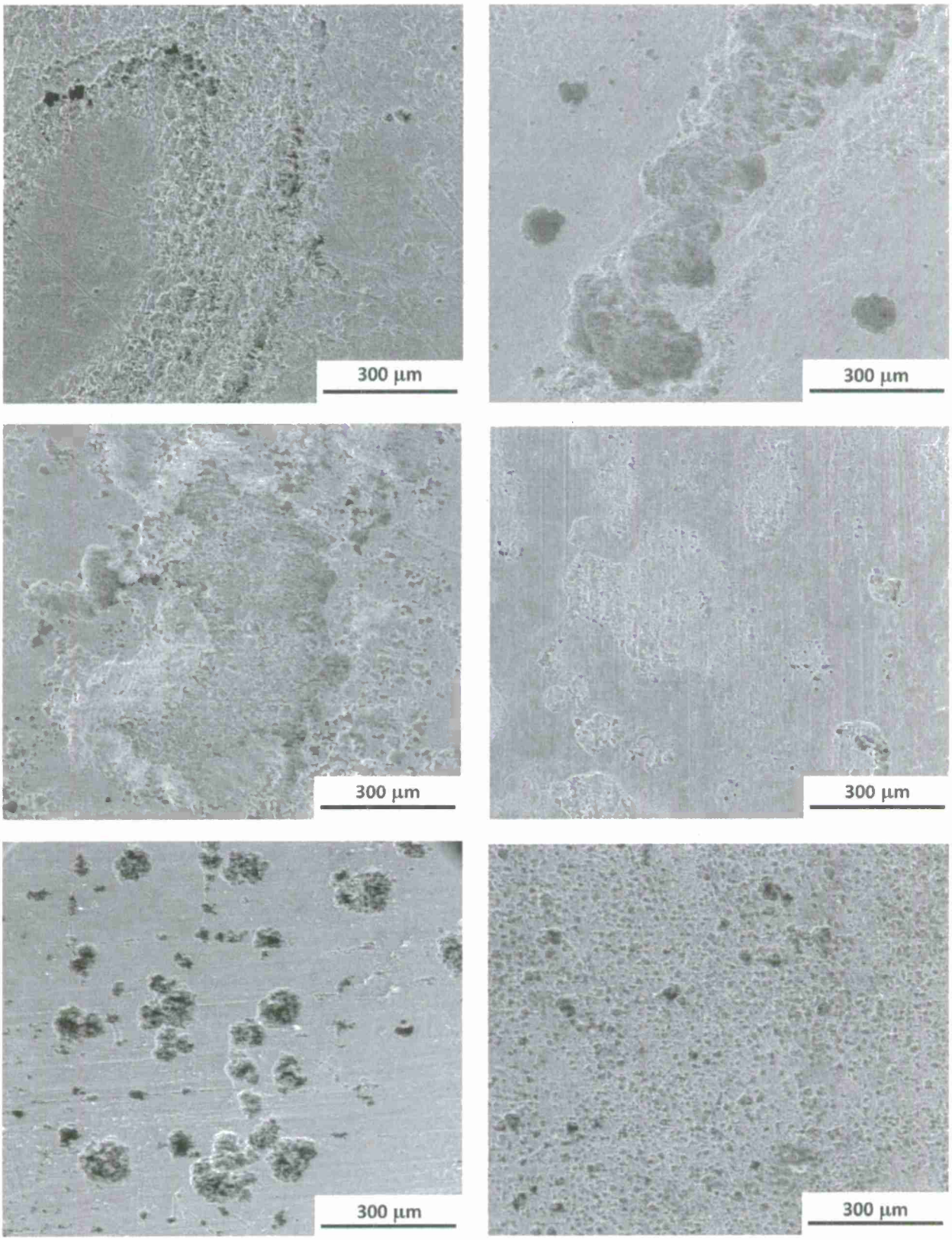

Figure 6. Corrosion morphology in carbon steel after 60 days exposure to FAME diesel in the presence of KW (left column) and PG (right column) seawaters. Exposures conditions include immersion in FAME (top row), seawater/FAME interface (middle row) and seawater (bottom row).

measured in KW seawater than in PG seawater (6 *vs* 2 ppm). Analysis of the black corrosion products by EDS on electrodes immersed in the FAME diesel layer indicated significant chloride and sulphide concentrations, suggesting these seawater ions diffused through the diesel and came into contact with the electrodes. These results indicate that seawater in the fuel accumulated at localized areas on the electrode surfaces and in combination with chloride and/or sulphide, caused pitting in the fuel phase. In general black corrosion products were associated with elevated sulphur as detected by EDS. The data presented here support the conclusion that corrosion was due to microbiologically produced sulphides. In both cases, the instantaneous corrosion rate increased with the onset of sulphide production as indicated by the distinct sulphide odor. Microscopic inspection of all carbon steel electrodes immersed in FAME diesel in contact with either seawater indicated pitting. However, pitting severity (ie depth) and extent (ie surface area) were most prevalent at the FAME diesel/seawater interface in the KW exposure.

Dissolved sulphides were measured after incubation of KW and PG seawaters with FAME diesel despite a low number of SRB. The relationship between numbers of microorganisms, particularly SRB, and MIC has never been established despite the development of numerous liquid culture test kits for their enumeration (Beech and Gaylarde 1999). Zhu et al. (2005) demonstrated substantial changes in the microbial population from a gas pipeline after samples were introduced into liquid culture medium. Using culture techniques, they reported that SRB dominated the microflora. However, using molecular analysis techniques similar to those used in the present study, the authors found that methanogens were more abundant in most pipeline samples than denitrifying bacteria and that SRB were the least abundant bacteria. The number of SRB in the original pipeline sample was 6.7×10^5 ml^{-1}, 1.5% of the total population.

Addition of FAME diesel altered the bacterial community in both seawaters, most notably increasing the relative abundance of 16S rRNA gene sequences affiliated with Clostridiales (Firmicutes) and that of the genus *Marinobacter*, suggesting there may be a characteristic microbial profile for seawater incubated with FAME diesel. The Clostridiales sequences were not similar to those of known sulphate- or thiosulphate-reducing Clostridiales. The increase in *Marinobacter* provides a plausible explanation for degradation of FAME diesel components. Strains of *Marinobacter* are noted for their ubiquity in marine systems, their ability to degrade a variety of hydrocarbons (Berlendis et al. 2010) and their rapid growth on oil and oil components (Yakimov et al. 2007). *Marinobacter* species are aerobic bacteria, but can also respire anaerobically, indicating that they may be of importance in degrading hydrocarbons at anoxic/oxic interfaces (Bonin and Bertrand 2000). However, it may be that these organisms are able to metabolize the fatty acids produced during the initial hydrolysis of component methyl esters. The production of a complex suite of fatty acids was found as methyl ester metabolites in an earlier study (Aktas et al. 2010).

The activity of the few SRB in the initial seawater incubations may have been limited, at least in part, by the availability of a suitable electron donor. In the present study, the rates for sulphate reduction in unamended seawater incubations were barely detectable. Amendment of an easily degradable carbon source (lactate, 10 mM), resulted in an order of magnitude increase in sulphate reduction in KW seawater and about a 2-fold increase in PG seawater. Thus, there was nothing fundamentally inhibiting sulphate reduction in the seawaters, but the resident microflora had a requirement for a suitable electron donor for sulphide production. Addition of FAME to KW or PG seawater incubation resulted in increased sulphide concentrations. A higher sulphide level was obtained in the KW (6 ppm) incubation than in the PG seawater (2 ppm). These present findings, combined with results by Lee et al. (2010a), suggest that KW seawater has a higher intrinsic potential for sulphide production than PG seawater under the same storage conditions. Not all organic sources are equally biodegradable. Some carbon sources can be used by very specific microbial populations. Thus, when KW and PG waters received crude oil as a source of organic matter, the rates of sulphate reduction did not appreciably change relative to *in situ* rates in substrate-unamended waters. Addition of a suitable hydrocarbon-degrading inoculum (*D. alkanexedens*, strain Lake) as well as crude oil resulted in a significant increase in the rate of sulphate reduction (Table 2). Sulphur cycling in ocean systems is complex, and includes the degradation of compounds such as dimethylsulphoniopropionate, which originate from a number of marine organisms (Keller et al. 1989; Gonzalez et al. 1999). Therefore, the rate of various sulphur transformations, which may depend in part on the availability of electron donors, may be more important for controlling the production of sulphide than the concentration of sulphate or absolute numbers of SRB.

Conclusions

Sulphide influenced corrosion rates of carbon steel exposed to seawaters and FAME diesel did not correlate with initial concentrations of sulphate,

chloride or organic carbon in the seawater. Microbial sulfide production was limited by availability of a suitable electron donor. A microbial community, dominated by Clostridiales (Firmicutes) and with a greatly increased abundance of hydrocarbonoclastic *Marinobacter* but with low numbers of SRB, developed after seawater was incubated with the alternative fuel. Plant-derived diesel stimulated sulphide production and corrosion in two coastal seawaters, but at different rates. The corrosion rates measured in the KW seawater incubation were three orders-of-magnitude higher than those measured in PG seawater. Significantly higher elemental concentrations of sulphur and chlorine (presumed sulphide and chloride) were detected in corrosion products in the KW exposure compared to PG. Initially higher estimates of Dsr- and Mcr-bearing cells (ie SRB and methanogens) in KW seawater compared with PG seawater provide the only indication that KW seawater will support more sulphate reduction. Over the 60 day exposure to FAME diesel, this difference is minimized. The inability to predict corrosivity of particular seawaters from a limited set of chemical and microbial parameters demonstrates that simple models in which SRB abundance is directly associated with rate or extent of corrosion are inadequate.

Acknowledgements

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14. ABSTRACT

Experiments were designed to evaluate the corrosion-related consequences of storing/transporting fatty acid methyl ester (FAME) alternative diesel fuel in contact with natural seawater. Coastal Key West, FL (KW), and Persian Gulf (PG) seawaters, representing an oligotrophic and a more organic- and inorganic mineral-rich environment, respectively, were used in 60 day incubations with unprotected carbon steel. The original microflora of the two seawaters were similar with respect to major taxonomic groups but with markedly different species. After exposure to FAME diesel, the microflora of the waters changed substantially, with Clostridiales (Firmicutes) becoming dominant in both. Despite low numbers of sulphate-reducing bacteria in the original waters and after FAME diesel exposure, sulphide levels and corrosion increased markedly due to microbial sulphide production. Corrosion morphology was in the form of isolated pits surrounded by an intact, passive surface with the deepest pits associated with the fuel/seawater interface in the KW exposure. In the presence of FAME diesel, the highest corrosion rates measured by linear polarization occurred in the KW exposure correlating with significantly higher concentrations of sulphur and chlorine (presumed sulphide and chloride, respectively) in the corrosion products

15. SUBJECT TERMS
seawater, carbon steel, FAME diesel, sulphide, MIC

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